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A method for measuring biomolecules

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ABSTRACT OF THE DISCLOSURE

This present invention is for a method referred to as the vessel transfer method that greatly simplifies the detection and determination of the identity, amount and activity of molecules with biological activity. It is concerned with use of a vessel that will facilitate and simplify the measurement of the amount and activity of enzymes, enzyme inhibitors, lectins, receptors and other biologically active substances using a vessel transfer for isolating a reactant from a product after completion of the reaction. The method involves a surface is coated with a reactant, the appropriate compounds are added, allowing the reaction to proceed for a given period of time and then reaction solution is transferred from the reaction vessel 1 coated with a reactant to the vessel 2 whose surface is not coated. The amount of labeled compounds in the vessel 2 can be directly measured.

BACKGROUND OF THE INVENTION

It is of great importance in all field and disciplines of the life sciences to utilize the appropriate qualitative and quantitative analytical techniques for the detection, identification, and measurement of the concentration of a wide variety of biologically important molecules. These analytical techniques can be utilized in many different types of assays including those for enzymes, receptors, lectins and inhibitors, etc.

All chemical reactions in living systems are virtually catalyzed by enzymes, and the assay of enzyme activity is probably one of the most frequently encountered procedures in biochemistry. Most enzyme assays are carried out for the purpose of estimating the amount or activity of an enzyme present in a cell, tissue, other preparation, or as an essential part of an investigation involving the purification of an enzyme. The current assay methods have been developed based on the physical, chemical and immunological properties where they can be detected using photometric, radiometric, high performance liquid chromatographic, electrochemical assays, etc. (Eisenthal, R. and Danson, M. J., 1993). Although the methods basically fulfill the many essential requirements for routine analysis, there are, among those, the varying disadvantages of low sensitivity (Brenda Oppert et al, 1997), multiple steps (Twining, S. S., 1994; Pazhanisamy, S. et al, 1995) and steps that are tedious and time-consuming (Fields, R., 1976). Immunoassays have been widely using in human clinical tests and therapeutics, agriculture, food, veterinary and environmental diagnostics (Deshpandes, S. 1996). In the most cases, immunoassays are effective and valid (Cleaveland, J. S. et al 1990), but in some cases they are not suitable, for example, in the determination of enzyme activity. This occurs because the

binding assays for antibody and antigen (enzyme) can only be used to measure the concentration of an antigen (enzyme) but not its activity. It is of important to know the catalytic activity of an enzyme and not just the amount of the enzyme as a given amount of the enzyme may have a widely varying activity depending on reaction conditions. Also antibodies tend to react only with structurally similar antigens such as a specific enzyme. Therefore, it is often not possible to quantitate the amount of an enzyme from a related species using immunoassays.

Pharmaceutical industries usually utilize conventional methods mentioned above to screen compounds for discovering drugs. This process is slow due to the several steps required and the large amount of compounds needed to be tested; on a good day, a lab might test 100 to 1,000 compounds. In the race to commercialization, pharmaceutical manufacturers are facing great pressure to reduce the time to discover new clinical drugs, cut assay costs, and screen more compounds and against more targets. Therefore, there is a very high demand to develop new methods to meet the requirements of a high throughput screening (HTS). Jones et al (1997) described a method using quenched BODIPY dye-labeled casein as a substrate for determining the activities of protease, which is sensitive and amenable to automation. The degree of quenching of the fluorescent tag is crucial in this method. If there is not enough quenching due to poor conjugate or degradation of the fluorescence-labeled substrate under storage, etc. the assay will not be very useful. Also this procedure has relatively high background values which reduce its sensitivity. Another example of a potentially useful high throughput assay was made by Marquardt, et al (PCT/US97/07983). The method involves many steps of coating wells of a microplate, washing the wells, adding biologically active substance to wells, washing the wells once more, adding the indicator enzyme to wells, washing the wells again and adding a color

development reagent. The assay cannot be readily used in assays requiring rapid analysis.

A new assay method not only having potentially excellent sensitivity but being suitable for high throughput assays is preferable. This invention outlines a procedure that can achieve these goals.

SUMMARY OF THE INVENTION

This invention provides a new method for the qualitative and quantitative analysis of bioactive substances. The assay method is based on a procedure for separating the reactants from the products (resultants) after completion of the reaction followed by measurement of the amount of a labeled reactant or labeled product that has been transferred into the vessel 2. The device consists of two parts, one of which is a reaction vessel 1 coated with a reactant and other is a vessel 2 without coating a reactant. The reaction begins by adding other reactants than those coated on the surface. The reaction will be stopped by transferring the reaction solution into the vessel 2. The amount of the labeled products or labeled reactant in the vessel 2 can be determined according to the intensity of its label which can be fluorescent, luminescent, chromogenic molecules, radioactive tag and enzymes, etc. The amount of the label in the reaction vessel 2 is directly or reciprocally proportional to the activity or amount of the bioactive substance that is to be measured.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for the detection, identification and measurement of the amount or activity of biologically active molecules via a separation of

reactants from products by transferring the reaction solution form the reaction vessel 1 to the vessel 2.

1. An assay in which the surface of a vessel 1 is coated with reactant 1 which is an acceptor of a moiety from reactant 3. Reactant 3 is a substrate for reactant 2 having a biological activity. When reactant 2 and reactant 3 are added into the reaction vessel 1, the labeled moiety from reactant 3 is transferred to reactant 1 due to the biological activity of reactant 2 acting on reactants 1 and 3. The amount of labeled reactant 3 transferred into the vessel 2 can be directly determined and is reciprocally proportional to the activity of reactant 2.

The reactions for the transfer of a moiety between reactant 1 and reactant 3 by reactant 2 is interfered with by reactant 4 which is an inhibitor of reactant 2. The amount of labeled reactant 3 transferred into the vessel 2 is directly proportional to the amount of reactant 4 (inhibitor).

2. An assay in which the surface of the vessel 1 is coated with reactant 1 which is a labeled substrate for reactant 2 having a biological activity. Addition of reactant 2 and other compounds essential for the reaction into the vessel 1 results in the initiation of a reaction in which labeled products of the reaction are released into the reaction vessel. The amount of the released label transferred into the vessel 2 can be directly measured and is proportional to the activity of reactant 2.

The reaction of hydrolyzation of reactant 1 coated on the surface of the vessel 1 by reactant 2 is interfered with by reactant 3 which is an inhibitor of reactant 2. The amount of the released label from reactant 1 transferred into the vessel 2 is reciprocally proportional to the amount of reactant 3 (inhibitor).

3. An assay in which the surface of the vessel 1 is coated with reactant 1 which is a binding ligand for product 1. Product 1 is formed from reactants 3 linked with either a label or a binding agent capable of binding specifically to reactant 1. Product 1 is caused by the biological activity of reactant 2 in a reaction vessel acting on reactant(s) 3. Product 1 and other non-reacted reactants after transferring into the vessel 1 to bind the binding agent in the vessel 1. The amount of labeled non-reacted reactant 3 transferred into the vessel 2 can be directly measured and is reciprocally proportional to the activity of reactant 2

The reactions of formation of product 1 from reactants 3 due to biological activity of reactant 2 is interfered with by reactant 4 which is an inhibitor of reactant 2. The amount of labeled non-reacted reactant 3 transferred into the vessel 2 is directly proportional to the amount of reactant 4 (inhibitor).

4. An assay in which the surface of the vessel 1 is coated with reactant 1 which is a binding ligand for reactant 3 being the labeled form of reactant 2. Competitive binding reactions between reactant 2 and reactant 3 for reactant 1 or between reactant 2 and reactant 1 for reactant 3 are initiated when a unknown amount of reactant 2 and a known amount of reactant 3 are added into the vessel 1. The amount of the labeled reactant 3 transferred into the vessel 2 can be directly measured and is directly proportional to the amount of reactant 2.

The reactions of the competitive binding between reactant 2 and reactant 3 for reactant 1 or between reactant 2 and reactant 1 for reactant 3 is interfered with by reactant 4 which is an inhibitor of reactant 2. The amount of the labeled reactant 3 transferred into the vessel 2 is directly proportional to the amount of reactant 4 (inhibitor).

EXAMPLES

The following examples are for an illustrative purpose only, and not to limit the scope of the invention.

Example 1

The assay of protein kinase A and its inhibitor

Materials: hydrolyzed and partially dephosphorylated casein, protein kinase 3' : 5'-cyclic AMP dependent (PKA), cyclic AMP, protein kinase inhibitor, Na_2HPO_4, NaH_2PO_4, NaCl, Tween-20 are from Sigma. ^{32}P-ATP is from New England Nuclear. The 96-well microplates are from VWR Canlab.

Method:

1. Preparation of a microplate 1:

(1). Casein dissolved in PBS to the concentration 5 ug/ml and 100 ul/well is added to each well of the microplate 1 and incubated at 37^0 C for 3 hr, and is then rinsed with PBST.

2. Protein kinase assay:

(1). A series of concentrations of protein kinase in phosphate buffer (PB pH 7.2, cAMP, ^{32}P-ATP) are added to the wells of the microplate 1 (100 ul/well). During incubation at 37^0 C for 30 min, ^{32}P is transferred from ^{32}P-ATP to casein by protein kinase. The reaction solution is then transferred into the microplate 2 to stop the reaction.

(2). The radioactivity of ^{32}P-ATP in the wells of the microplate 2 is counted in a scintillation counter and is reciprocally proportional to the activity of the protein kinase.

3. Protein kinase inhibitor assay:

(1). Varying concentrations of an inhibitor in the phosphate buffer (PB pH 7.2, cAMP, ^{32}P-ATP) are added to the wells (50 ul/well) of the microplate 1. Negative and positive control are also included.

(2). A fixed amount of protein kinase A (50 ul/well) is added to each well containing inhibitor and the controls. incubated at 37^{0} C for 30 min. Protein kinase catalyzes the transfer of ^{32}P from ^{32}P-ATP to casein with the degree of transfer being reciprocally related to the concentration of the inhibitor.

(3). The radioactivity of ^{32}P-ATP transferred into the wells of the microplate 2 is counted in a scintillation counter and is directly proportional to the amount of the inhibitor.

This assay is a example of the general assay given in section 1 of the "Detailed Description of the Invention".

Example 2

Fluorescent assay of protease and protease inhibitors

Materials: proteinase K, elastase, protease XIII, papain, trypsin, pepsin, casein, dimethyl sulfoxide (DMSO), Na_2HPO_4, NaH_2PO_4, NaCl, Tween-20, citrate, ovomucoid, aprotinin are from Sigma. NHS-coumarin is from Molecular Probes. The 96-well microplates are from VWR Canlab.

Method:

1. Preparation of a fluo-casein: 5 mg NHS-coumarin in 100 ul DMSO is mixed with 10 mg casein in PBS (pH 7.2) in a micro-centrifuge tube and incubated at room temperature for 3 hr.

2. Preparation of a microplate 1:

(1). Fluo-casein dissolved in PBS to the concentration 5 ug/ml and 100 ul/well is added to

each well of the microplate 1, incubated at 37° C for 3 hr, then is rinsed with PBST.

3. Protease activity assay:

(1). A series of concentrations of a protease in a buffer (100 ul/well) are added to the wells of a microplate 1 and incubated at 37° C or room temperature for 30 min. The fluo-casein is hydrolyzed by protease to release the fluorescent labels into the microplate 1. the reaction solution is transferred from the microplate 1 into the microplate 2 to stop the reaction.

(2). The fluorescent intensity of the label in the wells of the microplate 2 is measured with a fluorometer and is directly proportional to the activity of the protease.

4. Protease inhibitor assay:

(1). Varying amounts of the protease inhibitor in the buffer (50 ul/well) are added to the wells of a microplate 1. Negative and positive controls are included.

(2). A fixed concentration of the protease in the buffer (50 ul/well) is added to the wells containing inhibitor and the controls and incubated at 37° C or room temperature for 1 hr. The fluo-casein is cleaved by the residual activity of the protease after inhibition to release the fluorescent label into the reaction vessels. The reaction solution is transferred into the microplate 2 to stop the reaction.

(3). The fluorescent intensity of the label in the wells of the microplate 2 is measured with a fluorometer and is reciprocally proportional to the amount of the inhibitor.

This assay is a example of the general assay given in section 2 of the "Detailed Description of the Invention".

Example 3

Telomerase and its inhibitor assay

Materials: Tris-acetate buffer (pH 8.5), potassium acetate, β-mercaptoethanol, spermidine, $MgCl_2$, EDTA, streptavidin are from Sigma. dATP, dTTP, fluo-dGTP, telomerase S10, RNase, biotin-oligonucleotide primer may be purchased from Boehringer Mannheim or elsewhere. Inhibitor (7-deaza-dATP).

Method:

1. Preparation of a microplate 1:

Streptavidin or avidin dissolved in a buffer to the concentration 5 ug/ml and 100 ul/well is added to each well of the microplate 1 and incubated at 37^0 C for 3 hr, and is then rinsed with PBST.

2. Telomerase activity assay:

(1). A series of concentration of telomerase in a reaction mixture containing 50 mM Tris-acetate pH 8.5, 50 mM potassium acetate (KAc), 5 mM β-mercaptoethanol, 1mM spermidine, 1mM $MgCl_2$, 0.5-2 mM dATP, 0.5-2 mM dTTP, 1.5 uM fluo-dGTP, 1 uM biotin-oligonucleotide primer $(TTAGG)_3$, are added to wells of a microplate and the mixture is incubated at 30^0 C for 1 hr. The reaction of DNA synthesis is stopped by adding the stop solution (10 mM Tris-HCl, pH7.5, 230 mM EDTA and 100 ug/ml RNase) at 37^0 C for 15 min.

(2). Isolation of the fluorescent label-DNA with biotin-primer (fluo-DNA-biotin) from the reaction vessel is done by the binding of the fluo-DNA-biotin complex to the streptavidin (avidin)-coated microplate 1 after reaction solution has been transferred into the microplate 1.

(3). The fluorescent intensity of fluo-dGTP transferred into the microplate 2 is measured with a fluorometer and is reciprocally proportional to the activity of telomerase.

3. Telomerase inhibitor assay:

(1). Varying amounts of the inhibitor in the reaction mixture (50 ul/well) are added to the wells of a microplate.

(2). A fixed activity of telomerase in the reaction mixture (50 ul/well) is added to the wells containing an inhibitor and the controls. Incubate the wells of the plate at 30° C for 1-2 hr and stop the DNA synthesis with the stop solution.

(3). Isolation of the fluorescent label-DNA with biotin-primer (fluo-DNA-biotin) from the reaction vessel is done via the binding of fluo-DNA-biotin complex to the streptavidin (avidin)-coated microplate 1 after the reaction solution has been transferred into the microplate 1.

(4). The fluorescent intensity of fluo-dGTP transferred into the microplate 2 is measured with a fluorometer and is directly proportional to the amount of the inhibitor.

This assay is a example of the general assay given in section 3 of the "Detailed Description of the Invention".

Example 4

Competitive assay of E. coli K88 fimbriae, receptor and inhibitor

Materials: Na_2HPO_4, NaH_2PO_4, NaCl, dimethyl sulfoxide (DMSO) are from Sigma. NHS-fluorescein is from Molecular Probes. A mucus receptor from a piglet and E. coli K88 fimbriae are prepared in a laboratory. Inhibitors are from different sources.

Method:

1. Preparation of fluo-fimbriae: 5 mg NHS-fluorescein in 100 ul DMSO is mixed with 10 mg fimbriae in 1 ml PBS (pH 7.2) followed by incubation at room temperature for 3 hr.

2. Preparation of a microplate 1:

(1). Receptor dissolved in PBS (pH 7.2) to a concentration of 5 ug/ml and 100 ul/well is

added to each well of the microplate 1 and incubated at 37° C for 3 hr, and is then rinsed with PBST.

3. Receptor assay:

(1). A series of concentrations of the receptor in a buffer (50 ul/well) are added to the wells of a microplate 1.

(2). A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the receptor and the control and incubated at 37° C for 1 hr. Competitive binding reactions between the immobilized receptor and the free receptor (competitor) to the fluo-fimbriae will occur.

(3). The fluorescent intensity of the fluo-fimbriae transferred into the wells of the microplate 2 is determined using a fluorometer and is directly proportional to the amount of the receptor (competitor).

4. Fimbriae or E. coli numeration assay:

(1). A series of concentration of the fimbriae or E. coli cell in a buffer (50 ul/well) are added to the wells of a microplate.

(2). A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the fimbriae or E. coli and control wells and incubated at 37° C for 1 hr. Competitive binding reactions between the fimbriae or E. coli (competitor) and the fluo-fimbriae to the immobilized receptor will occur.

(3). The fluorescent intensity of the fluo-fimbriae transferred into the wells of the microplate 2 is determined using a fluorometer and is directly proportional to the amount of the fimbriae or E. coli cells (competitor).

5. Inhibitor of the fimbriae-receptor binding assay:

(1). A series of concentration of the inhibitor in a buffer (50 ul/well) are added to the wells of a microplate 1.

(2). A fixed amount of the fluo-fimbriae (50 ul/well) is added to each well containing the inhibitor and the controls and incubated at 37^0 C for 1 hr. The reactions between the inhibitor and the immobilized receptor for binding to fluo-fimbriae or between the inhibitor and the fluo-fimbriae for binding to the immobilized receptor will occur.

(3). The fluorescent intensity of the fluo-fimbriae transferred into the wells of the microplate 2 is determined using a fluorometer and is directly proportional to the amount of the inhibitor (competitor).

This assay is a example of the general assay given in section 4 of the "Detailed Description of the Invention".

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CLAIMS

We claim:

1. A method for measuring the activity or concentration of a biomolecule comprising:

providing a reaction vessel 1 coated with a reactant, said reactant being capable of interacting with the biomolecule;

adding a known quantity of compound with a detectable label and a sample, said compound and sample including a biomolecule having a biological activity into the reaction vessel 1;

providing a reaction vessel 2 which is not coated with a reactant;

transferring the reaction mixture from the reaction vessel 1 into the reaction vessel 2; and

measuring the quantity of detectable label in the reaction vessel 2 and/or in the reaction vessel 1.

2. The method according to claim 1 wherein the detectable label is selected from the group consisting of: colorimetric label; radioactive label; luminescent label; fluorescent label and enzymes.

3. The method according to claim 1 wherein the reactant is bound to the vessel 1.

4. The method according to claim 1 wherein the sample is a biological sample.

5. The method according to claim 1 wherein the biological activity is an enzymatic activity.

6. The method according to claim 1 wherein the biological activity is a binding affinity.

7. The method according to claim 1 wherein the sample includes an inhibitor of the biological activity of the biomolecule.

8. The method according to claim 1 wherein the sample includes a competitor of the biological activity of the biomolecule.

9. The method according to claim 1 wherein the biomolecule is selected from the group consisting of: an enzymatic product; an enzyme; an inhibitor; a substrate; a receptor; a receptor binding ligand; a lectin; a lectin binding ligand; an antigen; and an antibody.

10. A method for measuring the activity or concentration of a biomolecule comprising:

providing a reaction vessel 1 coated with a reactant, said reactant being capable of interacting with the biomolecule, said reactant linked with a label;

adding a sample, said sample including a biomolecule having a biological activity into the reaction vessel 1 such that the reactant with a label contact the biomolecule and interact with the biomolecule such that label is released from the reactant;

providing a reaction vessel 1 which is not coated with a reactant,

transferring the reaction mixture from the reaction vessel 1 into the reaction vessel 2; and

measuring the quantity of detectable label in the reaction vessel 2 and/or in the reaction vessel 1.

11. The method according to claim 11 wherein the detectable label is selected from the group consisting of: colorimetric label; radioactive label; luminescent label; fluorescent label and enzymes.

12. The method according to claim 11 wherein the reactant is bound to the vessel 1.

13. The method according to claim 11 wherein the sample is a biological sample.

14. The method according to claim 11 wherein the biological activity is an enzymatic activity.

15. The method according to claim 11 wherein the biological activity is a binding affinity.

16. The method according to claim 11 wherein the sample includes an inhibitor of the biological activity of the biomolecule.

17. The method according to claim 11 wherein the sample includes a competitor of the biological activity of the biomolecule.

18. The method according to claim 1 wherein the biomolecule is selected from the group consisting of: an enzymatic product; an enzyme; an inhibitor; a substrate; a receptor; a

receptor binding ligand; a lectin; a lectin binding ligand; an antigen; and an antibody.